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14. ABSTRACT
Adenovirus (Ad)-mediated transduction of dendritic cells (DCs) is inefficient because of the lack of the primary Ad receptor, CAR. CD40 is a surface marker expressed by DCs that plays a crucial role in their maturation and subsequent stimulation of T cells. DC infection with Ad targeted to the CD40 results in increased gene transfer. Cells transduced with CD40-targeted Ad5-SV40-TAg vector showed increased expression of transgene and expression of co-stimulatory molecules at 48 hours post-infection compared to cells transduced with untargeted Ad5-SV40-TAg vector. We demonstrated that CD40-targeted gene transfer promotes DC maturation with induction of a complex signaling cascade accompanied by characteristic changes in cyto-kine production. These results demonstrate that DCs can be successfully transduced using a CD40 targeted adenoviral vector and that transduced DCs show activation.

15. SUBJECT TERMS
ovarian cancer; gene therapy; dendritic cells; adenovirus; cd40; tumor antigen; vaccination; targeting; ctl response; antigen presenting cell; syngeneic tumor model; immunization; pre-clinical

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Introduction

Dendritic cells (DCs) capture, process and present antigens in association with MHC class I and class II molecules to naive CD8+ cytotoxic and CD4+ helper T cells. Through this, specific cytotoxic T cells are activated, and recognize a target cell and kill it. This study was to determine the transduction efficiency of DCs using a CD40-targeted adenoviral vector expressing a tumor antigen. Recently, we characterized a new model using a mouse ovarian carcinoma cell line (IG10) that we have engineered to express the SV40 large T Antigen and forms tumors in syngeneic mice. The SV40 large T-Ag is highly immunogenic, inducing both antibody and cytotoxic T lymphocyte (CTL) responses. Since this antigen is synthesized in IG10 cell clones, the SV40 large T-Ag is an attractive candidate as a model system for the development of a DC-targeted cancer vaccine. We hypothesize that transduction of DCs in vitro using a CD40-targeted Ad5 vector expressing SV40 TAg (Ad5-SV40 TAg) will result in a high level of transgene expression, and be effective in inducing an antigen-specific CTL response. To target Ad5-SV40 Ag to DCs, we utilized a recombinant adapter protein consisting of extracellular portion of the native adenovirus receptor, CAR, fused to a trimerization motif from T4 fibritin protein, and linked to the extracellular domain of the mouse CD40 ligand (Figure 1).

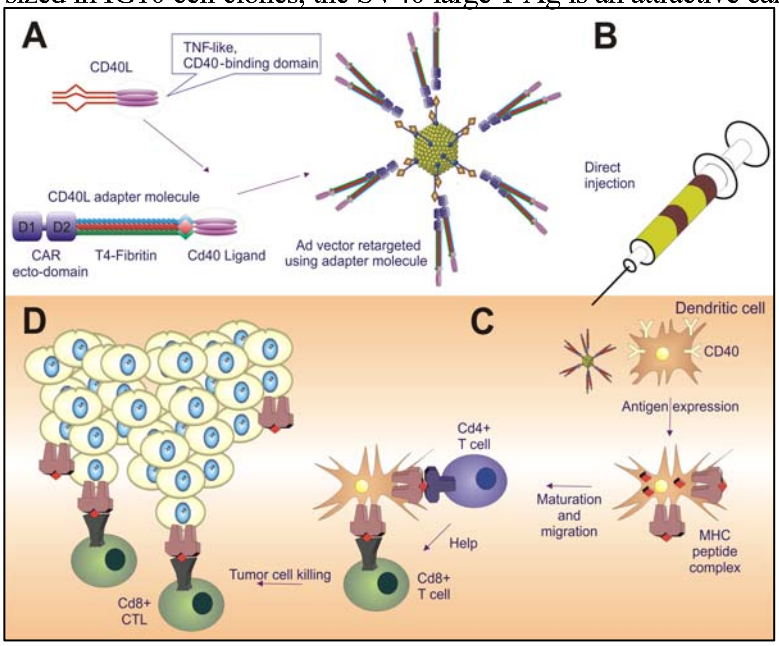

Figure 1. Targeting of Ad5 Vector to CD40 with TNF-Like Domain of CD40L Provides Flexible Platform for DC Transduction. The development of an in vivo approach based on DC vaccination without isolation and culturing of DCs ex vivo would be clinically significant. However, a critical component of in vivo transduction is efficient targeting of the vector to DCs without perturbation of DC function. To this end, we generated Ad vector systems that specifically target human and mouse DCs via the CD40 receptor using CAR-CD40L bi-specific adapter molecules **(A)** and showed that CD40-targeted Ads efficiently transduce DCs in vitro without interfering with DC function. We evaluated a dendritic cell-targeted Ad vaccine expressing the simian virus 40 (SV40) large T antigen (TAg) in a mouse model of ovarian cancer. We hypothesize that immunization of DCs **(B)** with the SV40 T-Ag will be effective in inducing antigen-specific cytotoxic T-lymphocyte (CTL) responses **(C)**, and suppress the growth of ovarian tumor cells expressing the SV40 T-Ag **(D)**.

Body

1. **Specific Aim I: Hypothesis: Transduction of DCs in vitro with a CD40-targeted Ad vector expressing the SV40 T-Ag [Ad5-(SV40 T-Ag)-CFm40L] will result in a high level of transgene expression, and be effective in inducing an antigen-specific CTL response in vivo.** In this Aim, we characterized phenotypic changes *in vitro* in isolated dendritic cells after infection with a CD40-targeted Ad vector compared to an untargeted Ad.

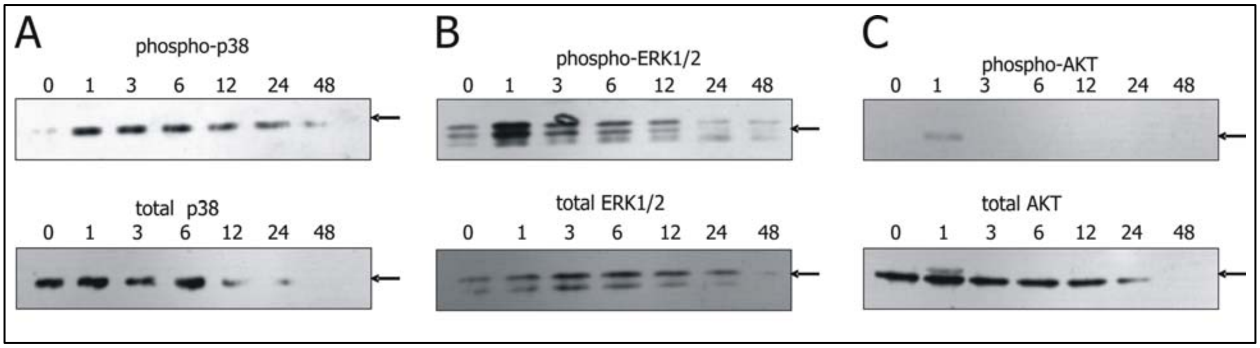

Figure 2. CD40L induces the phosphorylation of p38 MAPK, ERK, and Akt. Cultured DCs were exposed to CD40 targeted Ad-SV40 TAg at 100 M.O.I. for the indicated time intervals at 37°C and then subjected to Western blot analysis with specific Abs. The top panels show the detection of phosphorylated forms of p38 MAPK, AKT, and ERK, and the bottom panels show the same plots probed for total p38 MAPK, AKT, and ERK proteins, respectively, to demonstrate equal loading of samples.

A CD40 targeted adenovirus activates p38 MAPK, ERK, and Akt in DCs. Signal transduction via the MAPK such as ERKs and the p38 MAPK or Akt kinase, a downstream target of the PI3K, play important roles in cellular responses including cell proliferation, differentiation, and survival. CD40 ligation of immature DCs results in a potent maturational and survival stimulus. We therefore looked for activation of these signaling pathways in human isolated DCs in culture treated with a CD40 targeted Ad-SV40 TAg. Activation of PI3K, ERK, or p38 MAPK results in their phosphorylation. As shown in Figure 2, we found that within 1 hour of infection, Akt, ERK, and p38 MAPK were activated. This activation was short-lived for Akt, but persisted for ERK and p38 MAPK.

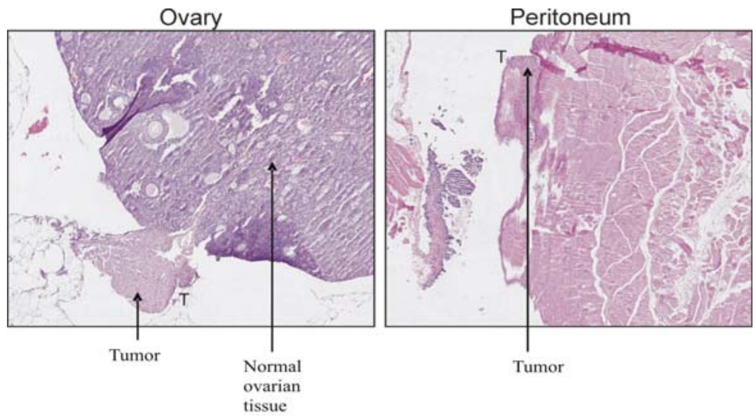

Figure 3. Tumor growth of IG10 cells *in vivo*. C57BL/6 mice were inoculated with 1 x 10^7 IG10 cells. The mice developed clinical tumor burden, observed as ascites fluid with gross carcinomatosis throughout the peritoneal cavity. Intraperitoneal tissues were paraffin embedded followed by H&E staining and light microscopy examination.

2. Specific Aim II: Hypothesis: Dendritic cells infected ex vivo with a CD40-targeted Ad5-SV40 TAg + CFm40L will generate protective tumor immunity in vivo against tumor cells expressing the SV40 T-Ag. In this Aim, we will establish a syngeneic immunotherapy model of ovarian cancer, and test the efficacy of an ex vivo vaccination approach. In addition, we will de-termine the ability of dendritic cells infected with Ad5-SV40 Tag + CFm40L *ex vivo* and transferred to host animals, to activate CTLs in vivo. Because of difficulties in producing consistent tumor growth using MOVCAR2 cells we investigated a model of mouse ovarian cancer generated by IG10 cells. In this model, we used a cell line developed using mouse ovarian surface epithelial cells obtained from normal mouse ovaries by mild trypsinization. These cells spontaneously transformed upon repeated passage in vitro, and showed a dramatic change in morphology and loss of contact inhibition. This process isolated one such cell line, the IG10.

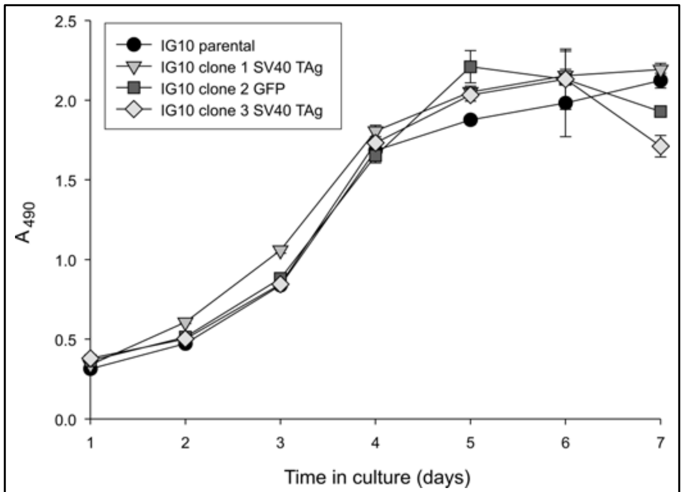

Figure 4. Growth assay of IG10 parental cells and cells stably transfected with SV40 Tag or GFP. The cells were plated into 96 well dishes at 10,000 cells /well. Beginning at day 1 and 24 h thereafter for a period of 7 days, one row of 12 wells from each plate was subjected to removal of medium and wash with PBS followed by drying. At the end of the growth assay, the number of cells in each row of each plate was determined by reading the absorbance at 490 nm after staining with 1% crystal violet, and comparing to a standard curve from the absorbance of known number of cells. Values represent the mean ± SEM of 12 replicate measurements.

Development of mouse tumor model of ovarian cancer. A total of 10 mice were used to develop a syngeneic peritoneal mouse model of ovarian cancer. After inoculation with the IG10 cells, the mice were monitored daily for tumor mass and evidence of sickness. Beginning with the eighth week after inoculation, the mice started to exhibit symptoms of distress such as altered gait, ruffled fur and extension of the abdomen and by the end of tenth week all mice in the study needed to be sacrificed. They were euthanized by asphyxiation with CO_2 and they were subjected to necropsy. The necropsy showed massive hemorrhagic ascites (approximately 10-15 ml each) and extensive tumor nodules (between 0.1 and 1 cm each) adherent to the surface of the peritoneum and on the surface of peritoneal organs: liver, stomach, intestine, spleen,

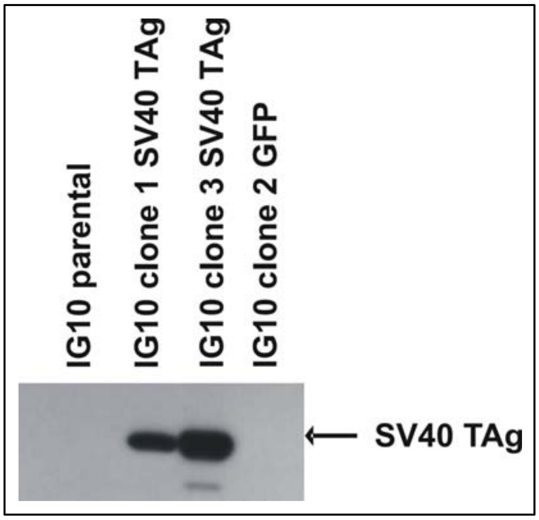

Figure 5. Western blot assay of IG10 cells. The expression of SV40 T-Ag is detected only in IG10 clonal cells that are stably transfected with an SV40 T-Ag expression plasmid.

ovaries or retroperitoneal ones such as kidneys. There were also visible non-adherent tumor masses in the peritoneal cavity. Pieces of adherent tumor together with adjacent organ tissue as well as pieces of non-adherent tumor masses were removed and they were subjected to histopathologic analysis by paraffin embedding and H&E staining of the sections. Light microscopy (Figure 3) examination revealed that the tumor masses consisted of a highly undifferentiated neoplasm, but the tumor was not penetrating the organs. This result demonstrates that the IG10 cell line can provide a useful model for peritoneal carcinomatosis which is similar with the advanced stages of ovarian cancer in humans. Gene therapy studies have used human ovarian cancer cell lines in order to identify strategies for gene transfer or to introduce specific therapeutic genes. These studies, carried on both in vivo and in vitro, had the disadvantage of requiring the use of immunodeficient mice. The conditions do not accurately model the human body and, consequently, the results might not represent the real outcome in a normal organism. The use of the IG10 mouse model of ovarian cancer can provide additional insights and reveal the real potential of different immunotherapy strategies by taking also into account various immune parameters. This fact, together with the possibility of using it in an immunocompetent mouse, constituted for us a strong reason to pursue with the use of this cell line in immunotherapy studies *in vivo*.

IG10 mouse ovarian cancer cell lines expressing SV40 TAg. The full-length SV40 TAg cDNA sequence was cloned into mammalian expression plasmid vector pEF1/V5-His downstream of the enhancer/promoter elements from the human elongation factor 1α subunit (hEF-1α) for high-level expression in mammalian cells. As a negative control, the green fluorescent protein (GFP) cDNA was also cloned into the pEF1/V5-His expression vector. The mouse ovarian cancer cell line IG10 was transfected with the expression vectors, and clonal stably transfected cell lines were isolated by G418 selection followed by limiting dilution. Three individual clones were isolated: IG10 clone 1 and IG10 clone 3 expresses the SV40 TAg and IG10 clone 2 expresses the GFP. In order to characterize the growth characteristics of the IG10 clones 1, 2, and 3 cell lines in culture, the cell numbers were measured over 7 days in culture. As shown in Figure 4, the results demonstrate that under normal growth conditions, all three IG10 cell lines grow in a similar fashion as the parental un-transfected IG10 cell line.

Western blot analysis of SV40 TAg expression. In order to determine the level of SV40 TAg expression in the IG10 clonal cell lines, Western blot analysis was performed. Lysates from the two IG10 clones stably transfected with the SV40 TAg cDNA (clone 1 and clone 3) were compared to the lysates from untransfected IG10 cells and IG10 cells stably transfected with GFP (clone 2). As shown in Figure 5, a predicted ~75 kD band was identified in the IG10 clone 1 and clone 3 but not in un-transfected IG10 cells or in IG10 clone 2 transfected with GFP. In the IG10 clone 1 and clone 3 cell lines, SV40 TAg was detected at similar strong levels of expression.

CTL assay of mouse splenocytes after administration of DCs treated ex vivo with CD40-targeted Ad vectors. To study the effect of CD40-targeted Ad transduction on DC-mediated CTL activation, we made use of the two CD40-targeted Ad vectors: Ad5-luc1 or Ad5-SV40 TAg.

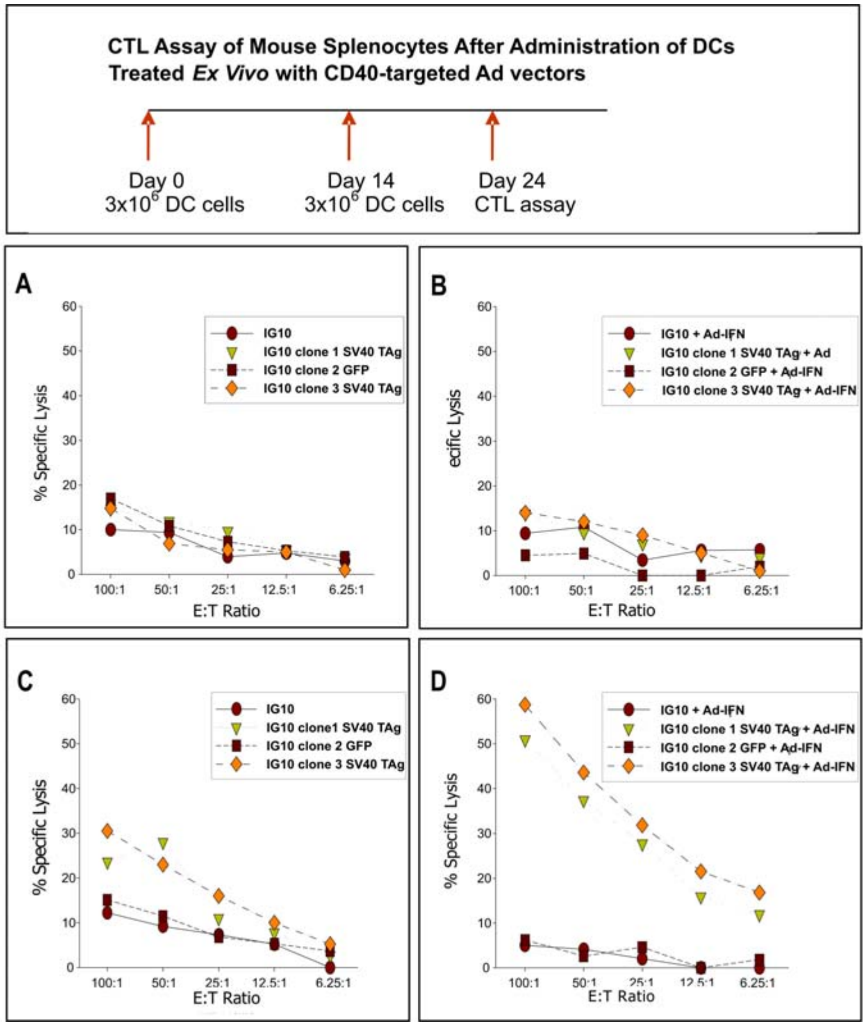

Figure 6. Chromium release assay of splenocyte activation in mice after adenovirus treatment. Top panel shows the dosing scheme for this experiment. Animals were treated with 3 × 10^6 DCs transduced *ex vivo* with either Ad5-luc1 (A, B) or with Ad5-SV40 TAg (C, D). Splenocytes from mice in the vaccination groups were used as effector cells against untreated IG10 target cells (A, C) or IG10 target cells treated by infection with Ad-IFN-γ (B, D). IG10 target cells included untransfected parental cells (●), IG10 clone 2 GFP (■), IG10 clone 1 SV40 TAg (▼), and IG10 clone 3 SV40 TAg (◆). After initiation of the experiment, the animals were killed, and cytolytic activity was measured. Results shown are the mean ± SE for triplicate cultures.

In the experiment shown in Figure 6, C57BL6 mice were immunized with 3 x 10^6 DCs transduced with either Ad5-luc1 or with Ad5-SV40 TAg. This immunization was repeated at 14 days, and at 24 days from initiation of the immunization regimen, the mice were sacrificed and the spleens were removed. Splenocytes from the mice were re-stimulated for 6 days with γ-irradiated 293 cells transduced with Ad5-SV40 TAg. Following re-stimulation, a classic ^{51}Cr release CTL assay was performed with the stimulated CTL as effector cells (E) and IG10 cells targets (T) at the indicated E:T ratios. Cytotoxicity was measured by ^{51}Cr release from IG10 target cells or IG10 clonal cell lines expressing SV40 TAg or GFP. Splenocytes from mice in the vaccination group receiving DCs transduced with Ad5-SV40 TAg exhibited specific lysis against IG10 clone 1 and clone 3 expressing SV40 TAg, but not against parental IG10 cells or IG10 clone 2 expressing GFP (Figure 6C). Splenocytes from mice in the vaccination group receiving DCs transduced with Ad5-luc1 did not acquire killing activity (Figure 6A). The differences between control group and the other two groups were statistically significant at E:T ratios of 100:1, 50:1, and 25:1 ($P < 0.01$).

Effect of interferon-gamma on CTL lysis. IFN-γ induces the expression of several components of the antigen-processing machinery, leading to enhanced presentation of peptides in the context of HLA class I molecules on the cell surface. This facilitates the T cell receptor–mediated

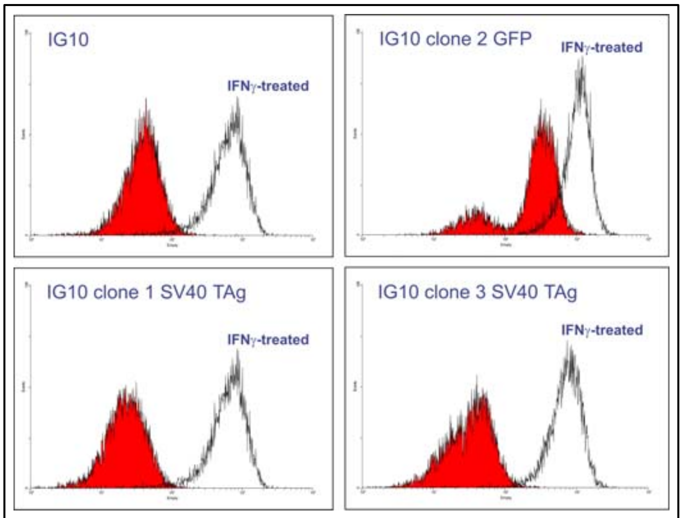

Figure 7. Flow cytometric analysis of MHC class I expression in IG10 cells maintained in the absence and presence of IFN-γ. Expression of MHC class I (H-2K^K) in IG10 parental and IG10 clonal cell lines engineered to SV40 TAg (clone 1 and clone 3) or the green fluorescent protein GFP (clone 2) was determined by flow cytometry analysis in uninfected cells or cells infected with Ad-IFN-γ.

(TCR-mediated) recognition of target cells by CD8+ T cells. Therefore we tested the effect of pre-treatment with an adenoviral vector expressing the murine interferon-gamma (Ad-IFN-γ) to enhance CTL lysis of target cells. As shown in Figure 6D, CTL reactivity was significantly enhanced in IG10 clone 1 and clone 3 expressing SV40 TAg, which were pre-treated for 24 hours with a 100:1 multiplicity of infection of Ad-IFN-γ compared to IG10 cells or IG10 clone 2 expressing GFP, which were also pre-treated for 24 hours with Ad-IFN-γ. However, specific lysis was not observed in any of the target cells despite pre-treatment with Ad-IFN-γ (Figure 6B).

Cell surface expression of MHC class IA molecules in response to IFN-γ. Cell surface expression of MHC class I molecules on IG10 cells in response to IFN-γ was determined. Basal cell surface expression of MHC class I was observed for IG10 and clone 1, 2 and 3 cell lines in the absence of Ad-IFN-γ treatment (Figure 7, A - D). Treatment with 100:1 multiplicity of infection of Ad-IFN-γ for 24 hours dramatically increased MHC class I cell surface expression on each of the IG10 cell lines compared with untreated control cells. The results of this experiment show that the IG10 and all of the clonal cell lines have low cell surface expression of MHC class I, while treatment of the cells with Ad-IFN-γ showed marked up regulation of MHC class I expression.

Western blot analysis of TAP1 and TAP2 protein expression in IG10 cell lines. To better characterize the antigen presentation pathway in IG10 cells, expression of TAP1 and TAP2 protein expression was studied by Western blot analysis. Antigenic peptides are generated in the proteasome of target cells, and then translocated across the membrane of the endoplasmic reticulum by the transporter associated with antigen processing (TAP). TAP is a trimeric complex consisting of

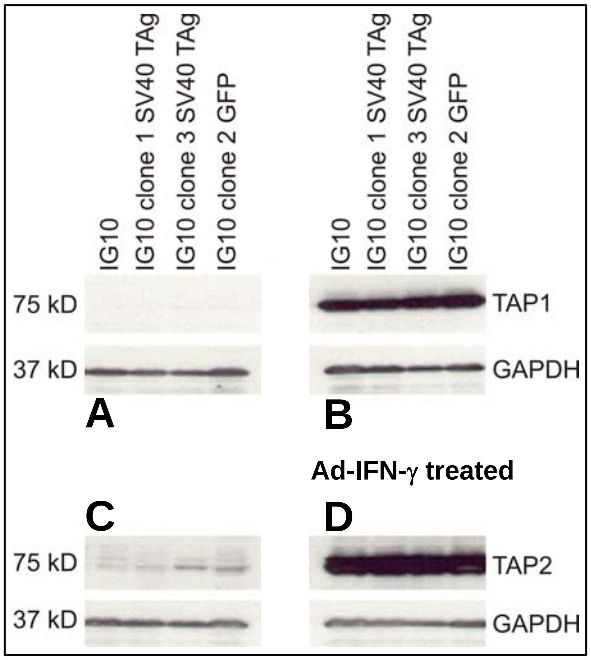

Figure 8. TAP Expression in IG10 Cell Lines is Induced by *Ad-IFN-γ*. Protein extracts (20 mg) were prepared from each cell line and diluted with RIPA sample buffer. The extracts were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis, electroblotted onto nitrocellulose and probed with an anti-mouse TAP1 antibody (A, B) or an anti-mouse TAP2 antibody (C, D), followed by treatment with corresponding anti-mouse IgG HRP conjugates.

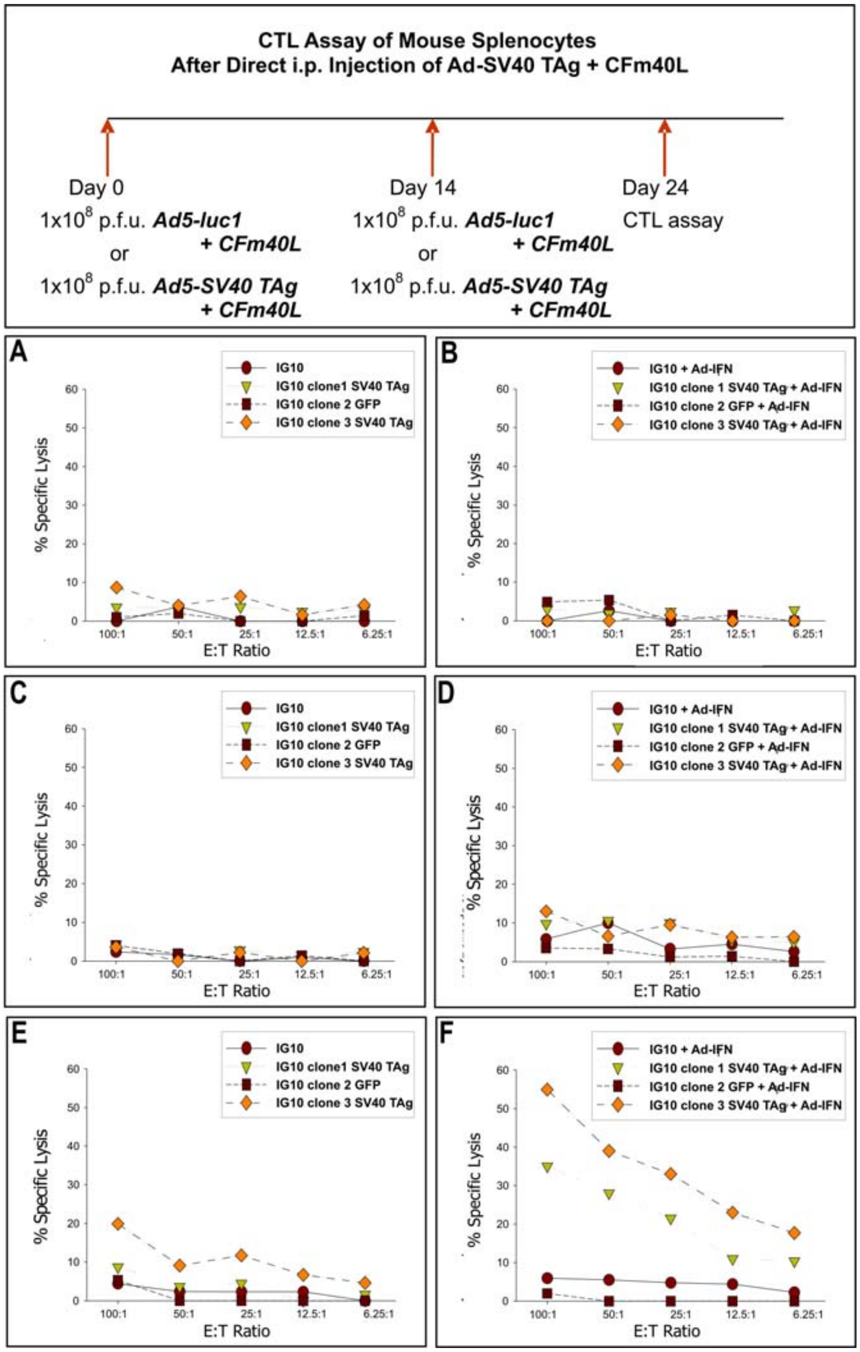

Figure 9. Chromium release assay of splenocyte activation in mice after adenovirus treatment. Top panel shows the dosing scheme for this experiment. Animals were treated with saline (A, B), 1×10^8 i.f.u. of CD40 targeted Ad5-luc1 (B,C, D) or CD40 targeted Ad5-SV 40 TAg. DCs transduced *ex vivo* with either Ad5-luc1 (A, B), or 1×10^8 i.f.u. of CD40 targeted Ad5-SV40 TAg (E, F). Splenocytes from mice in the vaccination groups were used as effector cells against untreated IG10 target cells (A, C, E) or IG10 target cells treated by infection with Ad-IFN-γ (B, D, F). IG10 target cells included untransfected parental cells (●), IG10 clone 2 GFP (■), IG10 clone 1 SV40 TAg (▼), and IG10 clone 3 SV40 TAg (◆). After initiation of the experiment, the animals were killed, and cytolytic activity was measured. Results shown are the mean ± SE for triplicate cultures.

TAP1, TAP2 and tapasin. As shown in Figures 8A and 8C, TAP1 protein levels were undetectable and TAP2 protein levels were barely detectable in any of the IG10 cell lines. However, treatment with 100:1 M.O.I of Ad-IFN-γ for 24 hours dramatically increased both TAP1 and TAP2 protein expression in each of the IG10 cell lines (Figures 8B and 8D) compared with the untreated cells. These results demonstrate that Ad-IFN-γ induces expression of the MHC class I antigen presentation pathway in IG10 cells.

CTL assay after direct intraperitoneal injection of Ad-SV40 TAg. The ultimate test of the functionality of the CD40-targeted Ad-SV40 TAg to deliver an antigen to DCs *in situ* was to assess DC-mediated CTL activation after direct intraperitoneal injection. As shown in the vaccination schema in Figure 9, C57BL6 mice were immunized intraperitoneally with 1 x 10^8 i.f.u. of either CD40 targeted Ad5-luc1 or CD40 targeted Ad5-SV 40 TAg. This immunization was repeated at 14 days with 1 x 10^8 i.f.u. of Ad, and at 24 days from initiation of the immunization regimen, the mice were sacrificed and the spleens were removed. Splenocytes from the mice were re-stimulated as described above, followed by a classic ^{51}Cr release CTL assay. Cytotoxicity was measured by ^{51}Cr release from IG10 target cells or IG10 clonal cell lines expressing SV40 TAg or GFP. As shown in Figure 9A, splenocytes from naïve mice exhibited no specific lysis against any of the IG10 cell lines. In, addition, no specific lysis was detected when the target cells were pre-treated with Ad-IFN-γ (Figure 9B). Similarly, when mice were vaccinated in situ with a CD40-targeted Ad5-luc1, no specific lysis against any of the IG10 cell lines, whether the target cells were pre-treated with Ad-IFN-γ or not (Figures 9C and 9D) In contrast, when mice were mice were vaccinated *in situ* with a CD40-targeted Ad5-SV40 TAg, clone 3 expressing SV40 TAg showed only weak CTL lysis, but no CTL lysis against parental IG10 cells or IG10 clone 2 expressing GFP, or against IG10 clone 1 expressing SV40 TAg (Figure 9E). As shown in Figure 9F, CTL lytic reactivity was significantly enhanced in IG10 clone 1 and clone 3 expressing SV40 TAg, when these cells were pre-treated for 24 hours with a 100:1 multiplicity of infection of Ad-IFN-γ compared to parental IG10 cells or IG10 clone 2 expressing GFP, which were also pre-treated for 24 hours with Ad-IFN-γ.

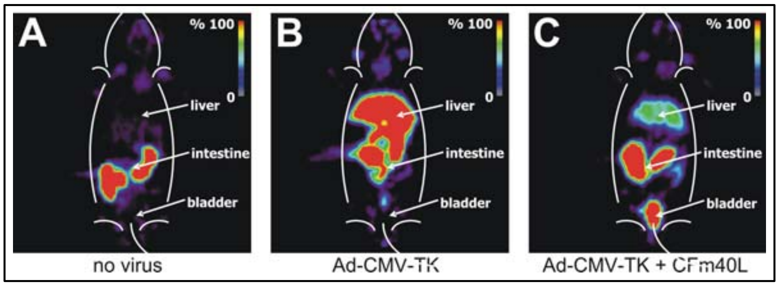

Figure 10. MicroPET imaging illustrating selective expression of HSV-Tk from the CD40 targeted Ad-CMV-HSV-Tk vector is restricted from normal liver compared to the untargeted Ad-CMV-HSV-Tk vector. (A) Image data from control mouse injected with 1.0 ml saline. (B) Image data from mouse injected intraperitoneally with 1 × 10^{10} infectious particles of untargeted Ad-CMV-HSV-Tk. (C) Image data from mouse injected intraperitoneally with 1 × 10^{10} infectious particles of CD40 taregetd Ad-CMV-HSV-Tk.

3. Specific Aim III: Hypothesis: In situ administration of a CD40-targeted Ad5-SV40 TAg + CFm40L will generate tumor immunity against IG10 cells tumor cells expressing the SV40 TAg. In this Aim, we will determine whether systemic administration of mice with a single dose of Ad5-SV40 Tag + CFm40L will result in protection against a challenge from a lethal dose of IG10 mouse ovarian tumor cells. This in vivo ap-proach will test efficacy of DC vaccination without isolation and culturing of DCs *ex vivo*.

Liver transduction of a CD40 targeted Ad *in vivo*. In order to determine the feasibility of this approach of CD40 targeting Ads and limiting cytotoxicity, herpes simplex virus thymidine kinase (HSV-Tk) expression was examined by microPET scanning, using [^{18}F]-FHBG as a substrate. In the experiment shown in Figure 10, three athymic nude mice were imaged. Image data from the control mouse injected with 1.0 ml saline (Figure 10A) showed only background accumulation of the [^{18}F]-FHBG substrate in the large intestine as well as urinary excretion in the bladder. Clinical toxicity of the Ad vector is primarily hepatic, based on the tropism of the virus to the liver. Intraperitoneal injection of a mouse with 1 × 10^{10} infectious particles of untargeted Ad5-CMV-HSV-Tk resulted in strong expression of HSV-Tk in the liver as demonstrated by accumulation of the [^{18}F]-FHBG (Figure 10B). Importantly, intraperitoneal injection of a mouse with 1 × 10^{10} infectious particles of CD40 targeted Ad5-CMV-HSV-Tk showed virtually no expression in the liver (Figure 10C). These results clearly show a differential expression between untargeted Ad-CMV-HSV-Tk and a CD40 targeted Ad-CMV-HSV-Tk in normal liver, and demonstrate that CD40 targeting can provide substantially lower liver transduction, which could limit associated liver toxicity.

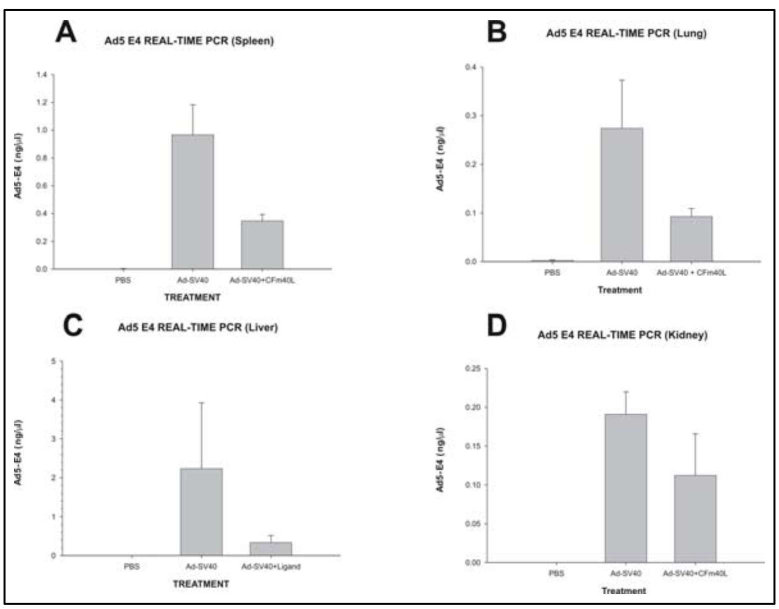

Figure 11. Evaluation of transduction *in vivo* of a CD40 targeted adenovirus. Viral copy number as an indicator of viral transduction in spleen (A), lung (B), liver (C), and kidney (D) obtained from five individual mice.

We further evaluated biodistribution *in vivo* of the CD40 targeted adenovirus compared to untargeted adenovirus. In this experiment intraperitoneal injection of five mice with 1 × 10^{10}

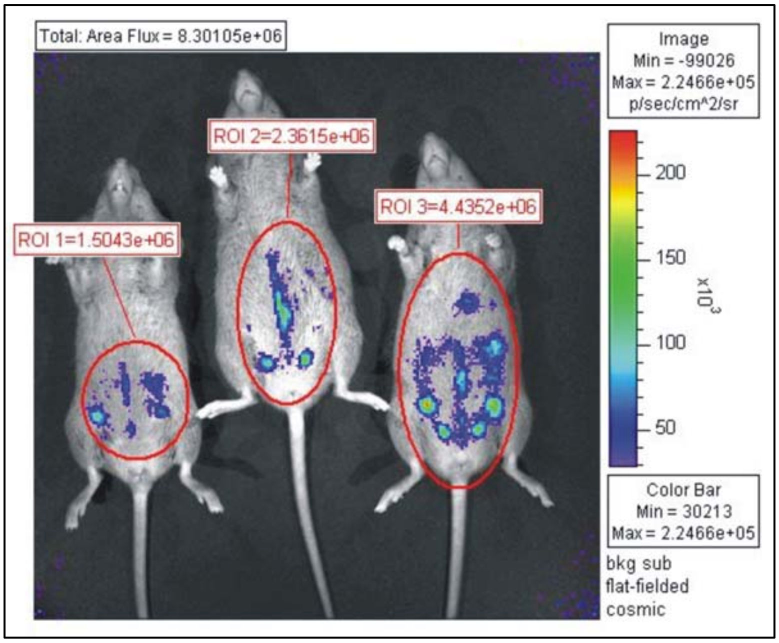

Figure 12. Bioluminescence imaging of mice. IG10 cells stably transfected with luciferase were injected intraperitoneally into syngeneic C57B6 mice. One week later the mice were administered the substrate D-luciferin and imaged.

infectious particles of untargeted Ad5-CMV-HSV-Tk was compared to intraperitoneal injection of five mice with 1 × 10^{10} infectious particles of CD40 targeted Ad5-CMV-HSV-Tk. To measure viral copy number, the presence of the E4 gene was determined using real-time PCR from DNA samples extracted from spleen (A), lung (B), liver (C), and kidney (D). As shown in Figure 11, liver and spleen showed the highest copy number among the tissues tested. Importantly, infection with the CD40 targeted Ad5-CMV-HSV-Tk resulted in significantly lower copy number. Thus, exploiting CD40 targeting allows a significant reduction of transduction in liver and other organs.

Estimating intraperitoneal tumor burden is difficult in vivo. To follow tumor growth *in vivo*, we developed clones of the IG10 cell line stably transfected with luciferase so that tumors can be imaged *in situ*. As shown in Figure 12, with this biolumiscent tool, we detected and visualized ovarian tumors. Thus, the luciferase-transfected tumor cells should enable an enhancement in the ability to follow tumor growth and metastasis in real time *in vivo*. In this following in vivo vaccination studies, we will use bioluminescent imaging to provide rapid, non-invasive monitoring of tumor growth and regression.

To elicidate anti-tumor immunity induced *in vivo* by administration of a CD40-targeted Ad. Our final task of this grant is to test the efficiency of the CD40-targeted ***Ad5-SV40 TAg + CFm40L*** in preventing the appearance of tumors upon IG10 challenge (protective) and in eradicating pre-established IG10 tumors (therapeutic).

Key Research Accomplishments

1. Changes in Maturation Profiles of Dendritic Cells Transduced with a CD40-Targeted Adenoviral Vector (2006) Disha A. Mody, Alexander V. Pereboev, Don A. Sibley, David T. Curiel, and J. Michael Mathis. American Society of Gene Therapy Ninth Annual Meeting. Abstract 836.
2. CD40-Targeted Adenoviral Vector Transduction of Dendritic Cells Disha Mody1, Don A. Sibley, Yoshinobu Odaka, Jagat Poduturri, Xiao L. Li, David T. Curiel, Alexander Pereboev, and J. Michael Mathis. American Society of Gene Therapy Tenth Annual Meeting. Abstract 451341.

Reportable Outcomes

Task 1

- We have characterized the phenotypic changes in p38 MAPK, ERK, and Akt signaling pathway activation *in vitro* in isolated dendritic cells after infection with a CD40-targeted Ad vector compared to an untargeted Ad.

Task 2

- We have established and characterized a syngeneic immunotherapy model of ovarian cancer using the IG10 mouse ovarian tumor cell line.
- We have determined the ability of dendritic cells infected with Ad5-SV40 TAg + CFm40L *ex vivo* and transferred to host animals, to activate CTLs *in vivo* against IG10 cells.
- We have characterized the induction of MHC II, TAP1 and TAP2 in IG10 cells after treatment with IFN-γ.
- We have determined the ability of dendritic cells infected with Ad5-SV40 TAg + CFm40L *in vivo*, to activate CTLs against IG10 cells.
- We have demonstrated enhance CTL killing of IG10 target cells by treatment with IFN-γ.

Task 3

- We have established determined the biodistribution *in vivo* of Ad5-SV40 TAg + CFm40L compared to untargeted Ad5-SV40 TAg.

Conclusions

- Western blot analysis of the phosphorylated forms of p38 MAPK, ERK, and Akt demonstrate activation of these signaling pathways by infection of dendritic cells with Ad5-SV40 TAg + CFm40L.
- Characterization of the IG10 cell line in syngeneic mice demonstrate that it can provide a useful model for peritoneal carcinomatosis which is similar to the advanced stages of ovarian cancer in humans.
- Western blot analysis of IG10 clones demonstrate stable transfection of the SV40 TAg.
- Splenocytes from mice in the vaccination group receiving DCs transduced with Ad5-SV40 TAg exhibited specific lysis against IG10 clones expressing SV40 TAg, but not against parental IG10 cells or IG10 clone 2 expressing GFP. However, administration of the SV40 TAg alone is not sufficient to stimulate a strong immune response.
- Pre-treatment with an adenoviral vector expressing the murine interferon-gamma (Ad-IFN-γ) enhances CTL lysis of IG10 target cells.
- Flow cytometric results demonstrate up-regulation of MHC II by pre-treatment with IFN-γ.

- Western blot assays demonstrate up-regulation of TAP1 and TAP2 by pre-treatment with IFN-γ.
- Splenocytes from mice in the vaccination group receiving Ad5-SV40 TAg +CFm40L exhibited specific lysis against IG10 clones expressing SV40 TAg, but not against parental IG10 cells or IG10 clone 2 expressing GFP. Pre-treatment with an adenoviral vector expressing the murine interferon-gamma (Ad-IFN-γ) enhances CTL lysis of IG10 target cells.
- Biodistribution of Ad5-SV40 TAg +CFm40L *in vivo* compared to untargeted Ad5-SV40 TAg demonstrated significantly lower liver transduction.

References

Appendices

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Changes in Maturation Profiles of Dendritic Cells Transduced with a CD40-Targeted Adenoviral Vector

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Introduction: Adenovirus (Ad)-mediated transduction of dendritic cells (DCs) is inefficient because of the lack of the primary Ad receptor, CAR. CD40 is a surface marker expressed by DCs that plays a crucial role in their maturation and subsequent stimulation of T cells. DC infection with Ad targeted to the CD40 results in increased gene transfer. Recently, we characterized a new model using a mouse ovarian carcinoma cell line (MOVCAR) that expresses the SV40 large T-Ag and forms tumors in syngeneic immunocompetent B6C3F1 mice. The SV40 large T-Ag is highly immunogenic, inducing both antibody and cytotoxic T lymphocyte (CTL) responses. Since this antigen is synthesized in MOVCAR cells, the SV40 large T-Ag is an attractive candidate as a model system for the development of a DC-targeted cancer vaccine. We describe the further characterization of the CD40-targeting approach using an adapter molecule that bridges the fiber of the Ad5 to CD40 on mouse DC. This adapter molecule, CFm40L, consists of the ectodomain of CAR genetically linked via a trimerization motif to the extracellular domain of mouse CD40 ligand. We have demonstrated that DCs transduced with a CD40-targeted Ad vector expressing SV40 T-Ag (Ad5-SV40 TAg) showed an increased expression of cell surface activation markers and were effective in inducing an antigen-specific CTL response. To examine CD maturation induced by transduction with the CD40-targeted Ad vector, we examined changes in a panel of cytokines and chemokines. In addition, we examined the kinase signalling pathways involved in cytokine regulation.
Methods: We used a Bio-Plex assay was performed on supernatants from untransduced DCs and DCs transduced with untargeted Ad and CD40-targeted Ad5 to determine changes in production of a panel of 23 cytokines and chemokines at 24, 48, and 72 hours post infection. We used a Kinexus phospho-antibody screening system on cell lysates from untransduced DCs and DCs transduced with untargeted Ad and CD40-targeted Ad to examine changes in phosphorylation / activation of 34 different protein phospho-kinases
Results: DCs transduced with a CD40-targeted Ad showed increased secretion of the cytokines IL-1A, IL-1B, IL-6, and IL-12, as well as chemokines MIP-1A and RANTES compared to untransduced DCs or DCs transduced with untargeted Ad. Similarly, DCs transduced with a CD40-targeted Ad showed a decreased secretion of cytokine KC. DCs transduced with a CD40-targeted Ad showed increased phosphorylation of MEK1, Erk1, Erk, and STAT1(T385), and decreased phosphorylation of Akt1, compared to untransduced DCs or DCs transduced with untargeted Ad. In contrast, DCs transduced with a CD40-targeted Ad showed an inhibition of Src, Gsk3a, Gsk3b, and STAT1(S727) phosphorylation compared to DCs transduced with untargeted Ad.
Conclusions: We demonstrated Ad-mediated CD40-targeted gene transfer to murine DCs using an adapter molecule CFm40L promotes DC maturation with induction of a complex signaling cascade accompanied by characteristic changes in cytokine production.
Keywords: Adenovirus; Immunotherapy; Targeted Gene Expression

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CD40-Targeted Adenoviral Vector Transduction of Dendritic Cells

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Introduction: Dendritic cells (DCs) are professional antigen presenting cells of the immune system. Targeting DCs with tumor antigens can result in DC-mediated presentation of the tumor antigen to the immune system and elicit tumor-specific immune response. DCs are difficult to transduce by using an adenovirus (Ad) vector because of the scarcity of CAR on the DC cell surface. CD40 is a cell surface marker expressed by DCs, is crucial for their maturation and the subsequent activation of the immune system by the DCs. We have explored the possibility of targeting DCs using Ad vector via CD40. We have used CFm40L, a bispecific adaptor molecule with ectodomain of CAR one end end linked genetically by a trimerization motif to the extracellular domain of mouse CD40 ligand on the other end. CFm40L bridges the fiber knob domain of Ad to CD40 on mouse DC. The SV40 T-Ag is known to be a highly immunogenic protein. We used SV40 T-Ag as a model antigen to test the efficacy of CD40-targeted Ad vaccine to tranduce DCs and generate an effective immune response against tumor cells bearing SV40 T-Ag.
Methods: Western blot analysis was used to detect expression of SV40 T-Ag in the DCs transduced with CD40-targeted and untargeted Ad-SV40 T-Ag. Chromium release assays were used to test the Cytotoxic T Lymphocyte response generated in the immunized B6C3F1 mice against cells bearing SV40 T-Ag. Morphological and histological examination of the liver was performed to compare the liver toxicity induced in B6C3F1 mice by CD40-targeted Ad-SV40 TAg and untargeted Ad-SV40 T-Ag. Real-time PCR analysis of Ad DNA in the liver samples was used to assess targeting of liver in B6C3F1 mice by the untargeted and CD40-targeted Ad. PET scans were performed to measure the expression of the transgene TK (thymine kinase) in the livers of B6C3F1 mice immunized with CD40-targeted and untargeted Ad expressing TK (*thymidine kinase*).
Results: 1) DCs transduced by using CD40-targeted Ad-SV40 T-Ag showed increased expression of SV40 T-Ag compared to untargeted Ad-SV40 T-Ag. 2) The B6C3F1 mice immunized with CD40-targeted Ad-SV40 T-Ag showed a greater Cytotoxic T Lymphocyte response than mice immunized with untargeted Ad-SV40 T-Ag. 3) There was reduced liver toxicity in B6C3F1 mice that were immunized with CD40-targeted Ad-SV40 T-Ag than the mice immunized with untargeted Ad-SV40 T-Ag as per morphological and histological examination. 4) CD40-targeted Ad vectors showed lower transduction of liver cells as compared to untargeted
Ad vectors as shown by real-time PCR analysis of Ad DNA and expression of transgene (TK) in liver samples of both groups.
Conclusion: These results demonstrated that CD40-targeted Ad vaccine is a more potent and a safer vaccine than the untargeted Ad vaccine.